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Lymphoid Procoagulant Response to Bacterial Endotoxin in the Rat

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A number of species respond to bacterial endotoxin (lipopolysaccharide [LPS]) wherein cells of the monocyte-macrophage lineage are rapidly induced either directly or via T-cell collaboration to initiate the extrinsic coagulation protease pathway. This results in fibrin formation and deposition as well as consumption of plasma coagulation proteins. It has been claimed that this cellular response, basic to the Shwartzman reaction, is lacking in rats and may account for the more limited severity of the Shwartzman reaction in this species. We examined the in vitro lymphoid procoagulant response in Fischer 344, Brown Norway, and Lewis rats. When peripheral blood mononuclear cells (PBM) were stimulated in vitro with LPS, a procoagulant activity (PCA) response was observed when assayed by acceleration of clotting of recalcified human or rat platelet-poor plasma. The response was rapid, with a maximum achieved at 4 h. PCA was not physically dissociated from viable PBM by 5 mM EDTA, which is consistent with the presence of an intrinsic plasma membrane initiator molecule rather than calcium-bound gamma-carboxylated glutamic acid-containing proteases. The induction of monocyte PCA was prevented by incubation of cells with cycloheximide or actinomycin D, implicating a new biosynthetic requirement. Cultivation of PBM with warfarin did not diminish the function of the effector PCA, nor did vitamin K augment the function of the endotoxin-induced PCA, indicating that the functional activity was not attributable to gamma-carboxylated glutamic acid-containing proteins. No inhibition of the cellular PCA molecule was produced by serine protease inhibitors. The LPS-induced PCA appeared to involve a tissue factor-like molecule since both factors X and VII were required in mediating PCA. Isolation of monocytes and T lymphocytes from LPS-stimulated PBM demonstrated that PCA was present in the monocyte-rich fraction. When isolated rat T lymphocytes and monocytes were separately exposed to LPS, PCA was not induced. In contrast, when the cells were combined, LPS induced PCA, indicating that the PCA response involved cellular collaboration between cells present in T lymphocyte and monocyte populations.

Disseminated intravascular coagulation (DIC) is a pathophysiological syndrome characterized by consumption of platelets and activation of coagulation factors, resulting in the deposition of multiple thrombi in various organs (18). It occurs in several pathological conditions, including trauma, malignancy, and infection by endotoxin-producing bacteria. The mechanisms underlying DIC are not fully understood. To obtain more information, attempts have been made to establish suitable animal models. It has been observed that different species respond with considerable variation to endotoxin-induced DIC, i.e., the Shwartzman reaction. The rat requires more than 10 times the quantity of endotoxin to induce DIC than, for example, the rabbit (1, 20, 29). Several different cells have been implicated to be responsible for the endotoxin-induced DIC, like platelets, endothelial cells, and leukocytes (4, 19). It has been suggested that this relative unresponsiveness to endotoxin-induced DIC in the rat might reflect a species-inherited impairment of selected leukocyte functions (24, 25).

We characterized the procoagulant activity (PCA) pathways underlying the cellular responses of humans and mice using peripheral blood mononuclear cells (PBMs) after in vitro stimulation with bacterial endotoxin (16, 23). It has been demonstrated that initiation of the coagulation protease cascade is mediated by monocytes but that induction or amplification of PCA is dependent on T-lymphocyte instruction (15, 21). The induced procoagulant effector molecule was in the human system of a tissue factor type (6) and in the mouse system of a prothrombinase type (6, 23). Others have also observed PCA by direct endotoxin stimulation of cells of monocyte lineage (7, 17, 26).

To investigate whether the relative in vivo refractoriness of the rat in regard to endotoxin-induced DIC is related to a lack of lipopolysaccharide (LPS) responsiveness of their lymphoid cells, we investigated the in vitro PCA response of rat PBMs to endotoxins. The results demonstrate that the rat, in response to endotoxin stimulation in vitro, indeed, can express monocyte procoagulant activity, but at a relatively low level compared with that of humans. The relative refractoriness in regard to the induction of DIC thus does not appear to represent an absolute deficiency in this cellular response; however, the quantitatively lower level of the response compared with other species may be related in part to the more limited pathology in this species. Thus, the rat does not represent an exception to the general monocyte-macrophage responses that initiate the coagulation protease cascade as an effector system and this should not be interpreted as a defect in lymphoid function.

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MATERIALS AND METHODS

Rats. Inbred female Fischer 344 rats were obtained from Simonsen Laboratories, Inc., Gilroy, Calif. Inbred female Brown Norway (BN) and Lewis rats were provided by the Research Institute breeding facility. The rats were maintained with water and chow ad libitum.

Cell preparations. Heparinized blood was obtained under aseptic conditions by cardiac puncture under ether anesthe-

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sia. Mononuclear cells were isolated on Ficoll-Hypaque (density, 1.074 g/ml) at 1,400 × *g* for 10 min followed by two washes in LPS-free RPMI 1640 medium (Irvine Scientific, Santa Ana, Calif.; prepared from powder by dissolving in pyrogen-free water) containing 2 mM L-glutamine and 50 μg of gentamicin sulfate per ml and suspended in medium containing 10% (vol/vol) heat-inactivated fetal bovine serum (Irvine). More than 98% of the cells were viable as determined by trypan blue exclusion. The proportion of monocytes in the PBM preparation were determined by the presence of cytoplasmic nonspecific esterase activity assayed by the method of Koski et al. (14); and in Fischer 344 rat PBMs it was 13.0 ± 3.9% (mean ± standard deviation [SD] of 21 cell preparations), in BN rat PBMs it was 4.3 ± 1.0% (mean ± SD of 4 cell preparations), and in the Lewis rat PBM it was 8.8 ± 3.0% (mean ± SD of 4 cell preparations).

Monocytes were isolated on the basis of their receptor-mediated attachment to plasma fibronectin (2, 10). Polystyrene petri dishes (20 by 100 mm) or 24-well tissue culture clusters (Costar, Cambridge, Mass.) were coated with 10 or 1 ml of LPS-free 2% gelatin (type II; Sigman Chemical Co., St. Louis, Mo.) in pyrogen-free water (Travenol Laboratories, Deerfield, Ill.) and incubated for 1 h at 37°C. The excess gelatin was then removed and the plates were dried for at least 4 h at 37°C. The plates were then stored at 4°C for up to a week. Before use, the plates were sterilized by exposure to UV light for 2 h. Fresh heparinized Fischer 344 rat plasma (5 or 1 ml, respectively) was then pipetted into each dish and incubated for 1 h at 22°C followed by 30 min at 4°C. The excess plasma was removed, and the plates were washed twice with RPMI 1640 medium. PBMs were incubated for 1 h at 37°C on the fibronectin-coated surfaces, and the nonadherent cells were removed by aspiration and five washes with a pipette using RPMI 1640 medium without serum supplement. Adherent cells were recovered by incubation at 4°C for 10 min with 3 mM EDTA. Monocytes obtained by this procedure were >90% nonspecific esterase positive and >96% viable as determined by trypan blue exclusion.

T lymphocytes were prepared by the passage of fibronectin nonadherent cells through nylon wool columns (13), in which 0.5 g of nylon wool (Fenwell Laboratories, Deerfield, Ill.) was packed into 12-ml disposable plastic syringes and used to fractionate 2×10^7 to 4×10^7 cells. After incubation for 1 h at 37°C, the T lymphocytes were eluted from the column with warm (37°C) RPMI 1640 medium supplemented with 10% fetal bovine serum. The effluent cells were <1% positive for nonspecific esterase activity and >98% viable.

PBMs were incubated for 4 h with or without LPS and then pipetted into the plasma fibronectin-coated 24-well tissue culture clusters (2×10^6 PBMs per well) and incubated for 1 h at 37°C (see Table 5). After incubation, the nonadherent cells were removed by aspiration and five washes with a pipette using RPMI 1640 medium without serum supplement. The T lymphocytes were prepared from the nonadherent cells as described above. The adherent cells were covered with 0.5 ml of 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)–0.15 M NaCl and stored at −70°C or, to estimate the amount of monocytes in the wells, stained for nonspecific esterase activity and counted.

Induction of PCA. PBMs, T lymphocytes, or monocytes at 10^6 per ml of RPMI 1640 medium supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Irvine) were incubated with LPS or medium at 37°C for 5 h in a humidified atmosphere of 5% CO_2–95% air. LPS used in these studies were from (i) *Escherichia coli* 0111:B4, (ii) *E. coli* 055:B5, (iii) *Salmonella minnesota* Re 595, or (iv) lipid A from *S. minnesota* Re 595 (Calbiochem Behring, La Jolla, Calif.). All cultures were done in triplicate. After incubation the cells were washed once, suspended in 0.5 ml of 25 mM HEPES in 0.15 M NaCl, and homogenized by three cycles of freeze-thawing and two cycles of sonication for analysis of total cellular PCA content. PCA expression by intact viable cells was determined on washed and suspended cells without homogenization. The cultivation of cells for PCA induction was performed in polypropylene tissue culture tubes (12 by 75 mm; Becton Dickinson Labware, Oxnard, Calif.).

Assay for PCA. In the PCA assay (15), 100 μl of cell suspension or homogenate and 100 μl of 20 to 25 mM $CaCl_2$ (depending on the plasma pool) were added to 100 μl of either pooled normal citrated human platelet-poor plasma or a platelet-poor citrated plasma pool from Fischer 344 rats. The clotting time, from the addition of plasma to formation of a visible clot, was measured in glass tubes with constant rocking at 37°C. The time was converted to milliunits of PCA per ml by reference to a standard curve derived from a rabbit brain thromboplastin standard (Difco Laboratories, Detroit, Mich.) at 37.5 mg (dry weight)/ml which was assigned a value of 10^5 mU/ml (15). Serial dilutions were used to produce a log-log plot. For reference, 10^3 mU of PCA per ml corresponds to a clotting time of approximately 50 s. The assay was not modified by the addition of cephalin with regard to net PCA, as the requisite phospholipid requirement was satisfied. Alternatively, the coagulation factor dependence was analyzed with congenital factor-deficient plasma samples from Daryl Fair (Research Institute of Scripps Clinic) and George King (Biomedical Inc., Overland Park, Kans.). Statistical analysis was performed by means of Student's *t* test.

Endotoxin contamination. All tissue culture media, fetal bovine sera, and chemical reagents used were tested for endotoxin contamination by the *Limulus* amoebocyte lysate assay (E-toxate; Sigma) and were negative at a level of 0.1 mg/ml.

Inhibition of protein synthesis. PBMs (10^6 cells per ml) were incubated for 1 h with 50 μg of cycloheximide per ml or 10 μg of actinomycin D (Calbiochem Behring) per ml prior to stimulation with LPS.

Pharmacological studies. Under conditions previously used to analyze biosynthesis (5), PBMs (10^6 cells per ml) were incubated for 24 h with or without vitamin K (Aqua Mephyton; Merck Sharp & Dohme, West Point, Pa.) or the warfarin derivative, 3-(α-acetonyl benzyl)-4-hydroxycoumarin (Sigma) prior to stimulation with LPS.

Protease inhibitors. LPS-stimulated Fischer 344 rat PBMs (10^6 cells per ml) were treated with the serine protease inhibitor diisopropylfluorophosphate (Sigma) at a concentration of 5×10^{-2}, 5×10^{-3}, 5×10^{-4}, or 5×10^{-5} M for 30 min at room temperature. The cells were then washed three times with 25 mM HEPES–0.15 M NaCl and tested for PCA. The effect of the serine protease inhibitor (*p*-amidinophenyl)methanesulfonyl fluoride (*p*-APMSF; California Medical Corp., San Francisco, Calif.) on the PCA expressed by homogenates of LPS-stimulated rat PBMs was also tested. The cell homogenates were tested with 10^{-2}, 10^{-4}, or 10^{-5} M *p*-APMSF at pH 7.0 for 30 min at room temperature. At this pH the half-life of *p*-APMSF is 6 min. After incubation the samples were tested for PCA. Homogenates or Fischer 344 rat thromboplastin, prepared from rat brains (8), were also treated for 30 min at 37°C with the cysteine

protease inhibitors mercuric chloride or iodoacetamide (both from Sigma).

RESULTS

When freshly isolated Fischer 344 rat PBMs were investigated the basal levels of PCA, a low level of activity comparable to that of normal human PBMs (6) was observed (Table 1). Of the total cellular PCA, approximately 31% was expressed by intact viable cells, which is consistent with cell surface localization of only a minor proportion of the activity. When the cells were incubated with LPS from *E. coli* 0111:B4, induction of PCA was observed. There was a 3.7-fold increase ($P < 0.001$) in total cellular PCA and a 1.7-fold increase ($P < 0.001$) in expression of PCA by intact viable cells as compared with control cells incubated in parallel in the absence of LPS (Table 1). This was a 5.9-fold ($P < 0.001$) and 2.1-fold ($P < 0.001$) higher PCA, respectively, than the basal activity of freshly isolated Fischer 344 rat PBMs that had not been cultivated in vitro. LPS-stimulated Fischer 344 rat PBMs were analyzed for PCA in both human and Fischer 344 rat plasma (Table 2). It was observed that the basal PCA and the maximum stimulated cellular PCA were apparently greater with rat plasma than with human plasma. The PCA, when assayed with either type of plasma, increased on incubation of the PBMs with LPS, demonstrating that the induced PCA or rat cells can be equally efficiently analyzed with human or rat plasma as substrate. For the following experiments cellular PCA was assayed with human plasma substrate.

Kinetics of the PCA induction by LPS. Incubation of 10^6 Fischer 344 rat PBMs with 50 μg of LPS per ml for various periods of time indicated that the total cellular PCA response was maximal after 4 h (Fig. 1). The induced PCA remained at elevated levels for up to 24 h of incubation (Fig. 1), after which it declined, reaching basal levels at about 72 h. The viable PCA showed the same kinetics, reaching its maximum after 4 h (data not shown).

Dose dependence of the PCA response to LPS. To identify the threshold for stimulation of Fischer 344 rat cells, 10^6 PBMs in 1 ml were incubated with increasing concentrations of LPS from *E. coli* 0111:B4 for 5 h and then assayed for total cellular PCA (Fig. 2). Exposure of cells to LPS at 1 μg/ml or less was associated with little induction of PCA but at higher concentrations in complete medium, induction of PCA was observed. The optimal stimulation was obtained with 50 μg of LPS per ml, and at higher LPS concentrations PCA decreased. Also, endotoxins from bacterial strains other than *E. coli* 0111:B4 were found to similarly induce PCA in Fischer 344 rat PBMs. LPS from *E. coli* 055:B5 and *S. minnesota* Re 595 all strongly induced PCA, as did the lipid A moiety of LPS produced by *S. minnesota* Re 595, indicating that the lipid A moiety rather than the polysaccharide are responsible for induction of PCA. For the rest of the study, LPS from *E. coli* 0111:B4 was used.

LPS-induced PCA in BN and Lewis rat PBMs. To investigate whether the LPS-induced PCA found in rat PBMs was restricted to the Fischer 344 rat strain, the response to LPS of BN and Lewis rat PBMs was investigated. PBMs from each of the rat strains were incubated with serially increasing

TABLE 1. Induction of PCA in Fischer 344 rat PBMs by endotoxin

Incubation time (h) and conditions of 2×10^6 PBMs per ml[a]	Viable cell PCA		Total cell PCA	
	Time (s)	mU[b]	Time (s)	mU[b]
0	130 ± 12	9 ± 3	110 ± 8	29 ± 8
5	124 ± 14	11 ± 3	91 ± 8	46 ± 16
5 + LPS	112 ± 9	19 ± 4	61 ± 5	172 ± 75

[a] A 5-h culture of 10^6 Fischer 344 rat PBMs per ml with or without 50 μg of LPS per ml. After incubation the cells were suspended in 0.5 ml of 25 mM HEPES in 0.15 M NaCl and either tested directly (viable PCA) or after freeze-thawing and sonication (total cell content) for the shortening of the spontaneous clotting time of recalcified human plasma.

[b] mU (mean ± SD) of PCA per 10^6 cells from seven experiments.

TABLE 2. PCA of endotoxin-induced Fischer 344 rat PBMs in human or Fischer 344 rat plasma[a]

Incubation time (h) and conditions of 2×10^6 PBM per ml[b]	PCA in human plasma		PCA in rat plasma	
	mU[c]	SI[d]	mU[c]	SI[d]
0	23 ± 8		64 ± 26	
5	39 ± 5	1.7	89 ± 40	1.4
5 + LPS	219 ± 44	9.5	506 ± 75	7.9

[a] Rabbit thromboplastin (333.3 mU/ml) gave a clotting time of 61 ± 6 s in human plasma and 57 ± 1 s in rat plasma.

[b] A 5-h culture of 10^6 Fischer 344 rat PBMs per ml with or without 50 μg of LPS per ml. After incubation the cells were suspended in 0.5 ml of 25 mM HEPES in 0.15 M NaCl.

[c] mU (mean ± SD) of PCA per 10^6 PBMs from three experiments.

[d] SI, Stimulation index, calculated by dividing the mU of PCA obtained with stimulus or medium by the base level mU of PCA.

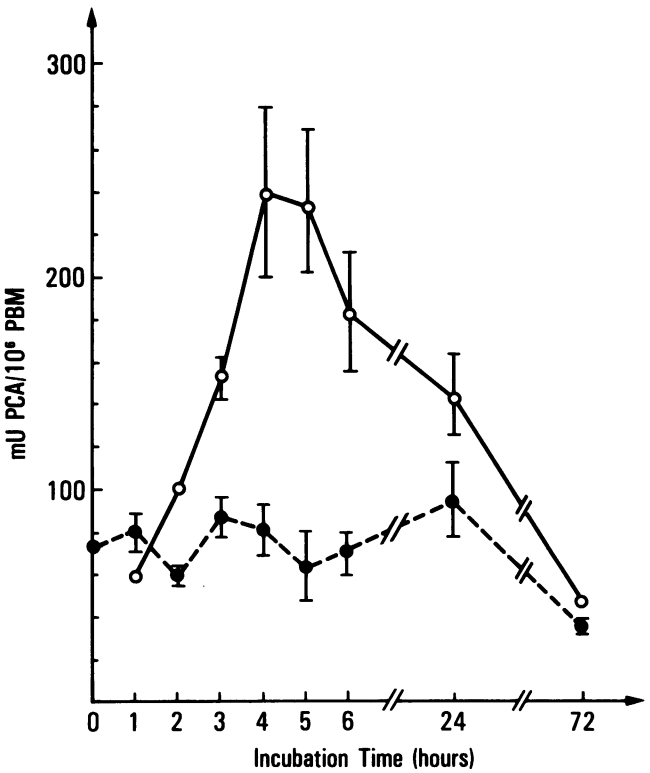

FIG. 1. Time dependence of LPS-induced PCA in Fischer 344 rat PBMs. Symbols: ○, 10^6 PBMs per ml incubated with 50 μg of LPS per ml for the indicated amount of time; ●, 10^6 PBMs per ml incubated for the indicated amount of time. The values are the mean ± SD of the mU of PCA per 10^6 cells.

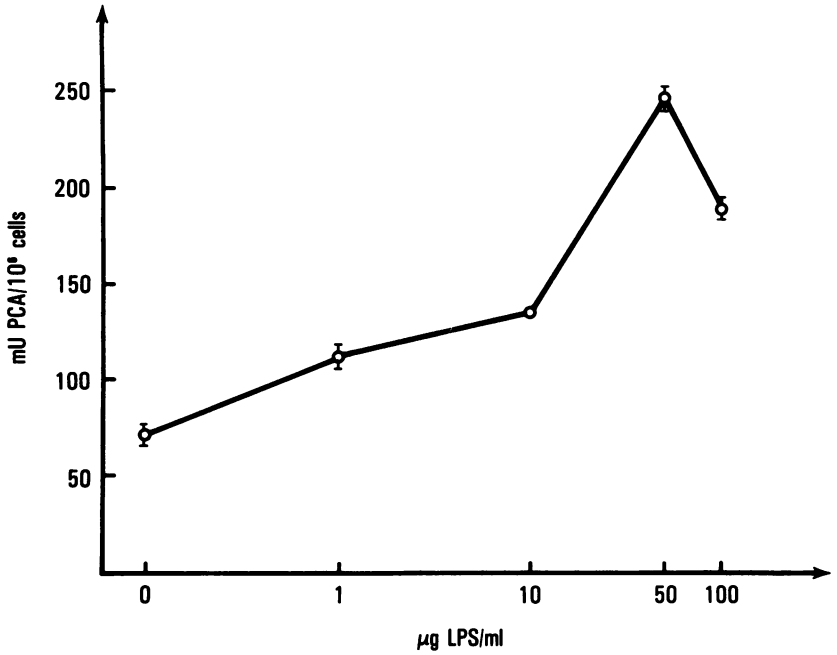

FIG. 2. Dose dependence of the LPS-induced PCA in Fischer 344 rat PBMs. A total of 10^6 PBMs per ml were incubated for 5 h with the indicated amounts of LPS. The values are the mean ± SD of the mU of PCA per 10^6 cells. PCA in control cultures without LPS was 55 ± 10 mU of PCA per 10^6 cells.

concentrations of LPS and analyzed for total cellular PCA. All three strains showed PCA responses to LPS stimulation. For example, the PCA obtained after stimulation with 50 μg of LPS was with Fischer 344 PBMs, 233 ± 58; BN PBMs, 215 ± 58; and Lewis PBMs, 109 ± 48 mU per 10^6 cells. The activity seen with Lewis PBMs was significantly lower ($P < 0.001$) than that obtained with Fischer 344 or BN PBMs. Also, the required concentration of LPS for maximum PCA induction was less for BN rat PBMs than for PBMs from the other strains (10 μg of LPS per ml as compared with 50 μg of LPS per ml for Fischer 344 and Lewis rat PBMs).

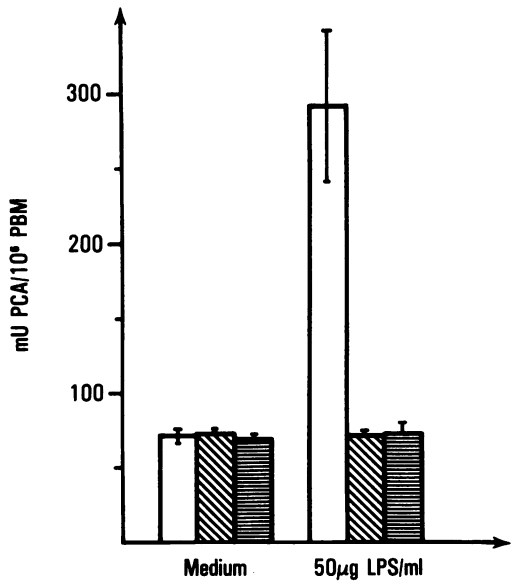

FIG. 3. Effect of cycloheximide and actinomycin D on LPS-induced PCA of Fischer 344 rat PBMs. Symbols: open bars, PBMs incubated with or without LPS; diagonally striped bars, PBMs treated with 50 μg of cycloheximide per ml and incubated with or without LPS; horizontally striped bars, PBMs treated with 10 μg of actinomycin D per ml and incubated with or without LPS. The values are the mean ± SD of the mU of PCA per 10^6 cells.

Cell surface expression of LPS-induced Fischer 344 rat lymphoid PCA. To investigate whether PCA expression by the stimulated Fischer 344 rat PBMs was attributable to calcium-dependent coagulation proteins such as the tissue factor-VIIa complex or the prothrombinase complex bound to the cells, cells were washed and incubated for 10 min at 37°C with 5 mM EDTA to dissociate bound calcium ions, and thus gamma-carboxylated proteins, and were then analyzed. The use of this concentration of EDTA is necessary for the chelation of tightly bound calcium ions involved in the tissue factor-VII/VIIa complex (28). LPS-stimulated Fischer 344 rat PBMs and control PBMs were incubated with EDTA, and following recovery of the viable cells by centrifugation, cell surface PCA was determined. No significant effect on LPS-induced PCA was observed, indicating that the PCA effector molecule is associated with the cell surface by other than divalent ions, consistent with an intrinsic membrane protein.

Metabolic requirements for expression of LPS-induced Fischer 344 rat lymphoid PCA. To further characterize the cell biology of PCA, the effect of the protein synthesis inhibitor cycloheximide or the RNA synthesis inhibitor actinomycin D on LPS induction of PCA was examined. Fischer 344 rat PBMs (10^6 cells per ml) were incubated for 1 h with 50 μg of cycloheximide or 10 μg of actinomycin D per ml prior to the addition of LPS. After 5 h of incubation, the total cellular PCA was determined (Fig. 3). Both drugs completely abrogated the LPS-induced PCA response, demonstrating that the response is dependent on new RNA and protein biosynthesis. To test a possible inhibitory effect of the drugs on the coagulation assay, rabbit thromboplastin

TABLE 3. Effect of warfarin and vitamin K on Fischer 344 rat PCA

Treatment (μg/ml)[a]	PCA[b]					
	Basal level		Medium		LPS	
	mU[c]	SI[d]	mU[c]	SI[d]	mU[c]	SI[d]
Untreated	49 ± 2		51 ± 3	1.0	111 ± 16	2.2
Warfarin						
0.1	36 ± 5		46 ± 6	1.3	106 ± 33	2.9
1.0	45 ± 1		41 ± 4	0.9	83 ± 18	1.8
10	23 ± 4		25 ± 5	1.1	49 ± 3	2.1
Vitamin K						
1.0	32 ± 2		32 ± 2	1.0	66 ± 1	2.1
10	27 ± 6		31 ± 9	1.2	55 ± 1	2.0

[a] 10^6 Fischer 344 rat PMBs per ml were incubated with or without various amounts of warfarin or vitamin K for 24 h at 37°C. Prior to stimulation with LPS, the cells were washed in medium containing the respective drug, and the viability was determined by trypan blue exclusion, revealing that all the cell preparations contained >90% viable cells.

[b] 10^6 Fischer 344 rat PBMs per ml were incubated with or without 50 μg of LPS per ml for 5 h.

[c] mU (mean ± SD) of PCA per 10^6 cells.

[d] SI, Stimulation index, calculated by dividing the mU of PCA per 10^6 cells obtained with stimulus or medium by the base level mU of PCA per 10^6 cells.

was diluted with buffer containing 50 μg of cycloheximide or 10 μg of actinomycin D per ml and tested. No effect on the shortening of the coagulation time was seen in the presence of the drugs, as compared with buffer alone. For example, 333 mU of thromboplastin per ml gave a 77% reduction of the clotting time. In the presence of cycloheximide the reduction was 76%, and with actinomycin D it was 74%.

Effect of vitamin K and warfarin on LPS-induced cellular PCA. To investigate whether the PCA induced by LPS in Fischer 344 rat PBMs was a vitamin K-dependent protein possessing gamma-carboxylated glutamic acid residues central to the function of these enzymes, rat PBMs were cultivated for 24 h in the presence of vitamin K or the vitamin K antagonist warfarin (3, 9, 12, 27). After stimulation with LPS, total cellular PCA was determined (Table 3). Cells cultivated with warfarin as well as those cultured with vitamin K expressed lower basal PCA than untreated cells. There was no evidence of decreased viability under these conditions previously utilized for analyses of cellular biosynthesis of the gamma-carboxylated coagulation proteases (5). On stimulation with LPS, however, the drug-treated cells responded equally well as untreated cells, giving stimulation indices of about 2. When the cells were cultivated with a mixture of vitamin K and warfarin (0.1 and 12.5 μg/ml, respectively) the same results as given above were obtained. A depression of the basal levels of PCA was seen in the cells treated with the drugs (47 ± 3 mU of PCA per 10^6 cells as compared with 82 ± 5 mU of PCA per 10^6 for untreated cells). After stimulation with LPS, however, both drug-treated and untreated cells responded with increased PCA, giving stimulation indices of about 2. These results indicate that the cellular PCA molecule is itself not a vitamin K-dependent protein, nor is the extrinsic coagulation cascade synthesized and assembled on the cell.

Effect of protease inhibitors on the induced PCA. To investigate whether the PCA expressed by stimulated rat PBMs was a serine protease, as has been described for the murine monocyte prothrombinase (23), LPS-stimulated Fischer 344 rat PBMs were incubated with the serine-protease inhibitors diisopropylfluorophosphate or *p*-APMSF at pH 7.0 (at which the half-life of *p*-APMSF is 6 min). Since the homogenates were incubated for 30 min, less than 1.5% *p*-APMSF activity remained at the time of assay for PCA. No effect of the serine protease inhibitors on PCA was observed. When PCA-positive cell homogenates were exposed to mercuric chloride in a range of 0.01 to 1 mM for 30 min and assayed, inhibition of PCA was observed. For example, with 0.1 mM mercuric chloride, an 82% inhibition of LPS-induced PCA was observed. However, some inhibition of the rat brain thromboplastin activity was also seen, indicating a possible nonspecific effect of the mercuric chloride. Furthermore, no inhibition was obtained when iodoacetamide was tested in a range from 0.01 to 100 mM, arguing against the possibility that rat PCA is of a cysteine protease nature as described by Gordon and Cross (11).

Characterization of the procoagulant activity induced by LPS. To examine functional characteristics of the procoagulant effector molecules produced by the LPS-stimulated PBMs, one-stage coagulation assays were performed with plasma substrates selectively deficient in single known coagulation proteins. Homogenates from LPS-stimulated PBMs were competent to accelerate coagulation of plasmas deficient in functional factors IX and VIII (Table 4). In contrast, the cell homogenates did not accelerate the coagulation of factor X- and VII-deficient plasmas, indicating a requirement for these proteases in mediation of the PCA, which is consistent with the presence of tissue factor. The acceleration of clotting observed with unstimulated cells is possibly due to a nonspecific effect of the homogenate, which contains both proteolytic activity and phospholipids. Interestingly, this nonspecific activity seems to be dependent on factors VIII and IX as well as factors VII and X.

TABLE 4. PCA of Fischer 344 rat PBMs stimulated with endotoxin with coagulation factor-deficient plasmas

Stimulus	Plasma clotting time (s) in the following[a]:				
	Normal human plasma	Factor-deficient plasmas			
		X	IX	VIII	VII
PBM (0 h incubation)[b]	87 ± 1	184 ± 5	186 ± 2	200 ± 5	117 ± 2
PBM (5 h incubation)[b]	87 ± 2	184 ± 2	186 ± 3	185 ± 2	114 ± 3
PBM (5 h incubation) + LPS[b]	66 ± 1	178 ± 1	80 ± 2	90 ± 3	120 ± 2
Thromboplastin[c]	68 ± 1	180 ± 7	67 ± 2	68 ± 2	136 ± 4
Buffer	218 ± 1	>360	>360	>360	220 ± 5

[a] Mean ± SD clotting time from a one-stage coagulation assay with human plasma samples deficient in the indicated factors.

[b] 10^6 Fischer 344 rat PBMs per ml were incubated with or without 50 μg of LPS per ml for 5 h.

[c] 100 mU of rabbit brain thromboplastin (tissue factor) per ml.

TABLE 5. PCA of cell populations isolated from Fischer 344 rat PBMs after stimulation with LPS

Cell population	PCA (mU/10^6 cells) in[a]:	
	Control	LPS-stimulated cells
PBM[b]	65 ± 5	306 ± 34
T lymphocytes[c]	18 ± 2	18 ± 1
Monocytes[d]	32 ± 14	630 ± 272

[a] 10^6 cells per ml were incubated with or without 50 μg of LPS per ml, and the monocytes (adherent cells) were obtained by adherence. The nonadherent cells were nylon wool fractionated to give T lymphocytes.
[b] 3.7% nonspecific esterase-positive cells.
[c] <1% nonspecific esterase-positive cells.
[d] 98% nonspecific esterase positive cells; 9% of the the total esterase-positive cells that were present in PBM.

Requirement of cellular collaboration for LPS-induced Fischer 344 rat PCA. To investigate the cell populations involved in the recognition as well as the biosynthetic response to LPS, Fischer 344 rat PBMs were incubated with or without LPS and then fractionated into adherent monocyte-enriched and nonadherent fractions. Most rat monocytes do not adhere well and are lost into the nonadherent fraction. The nonadherent fractions were fractionated on nylon wool columns, giving a relatively highly enriched T-lymphocyte fraction. The two relatively highly enriched cell populations thus obtained were used to examine the requirements for induction of cellular PCA. A representative experiment is presented in Table 5. The T-lymphocyte preparations from the LPS-stimulated PBMs did not increase in PCA content as compared with unstimulated control cells, whereas an increase of PCA was observed in the adherent monocyte population derived from the LPS-induced PBMs. The residual activity of the nonadherent population was removed with nylon wool and is not reflected in the lymphocyte population. The less adherent monocyte may be the most responsible; however, current methods of cell isolation have not yet permitted a definitive analysis. Thus, the LPS-induced rat PCA is expressed by monocyte populations as has been demonstrated for other species (6, 15, 17, 26).

To investigate whether T lymphocytes participate in the induction of the monocyte procoagulant response, Fischer 344 rat PBMs were fractionated by fibronectin-mediated adherence, to yield a monocyte-enriched population (fibronectin-adherent cells, >90% nonspecific esterase-positive cells) and a T-lymphocyte-enriched population (fibronectin and nylon wool nonadherent cells, >99% nonspecific esterase-negative cells). The separate cell populations were then tested for a PCA response to LPS (Table 6). Neither the monocytes alone in this or more than 16 other experiments nor the T lymphocytes alone generated a significant increase of PCA in response to LPS. When the cell populations were combined in a ratio of 10:1 (T lymphocytes to monocytes) and stimulated with LPS, a 1.5-fold increase in PCA as compared with unstimulated but incubated control cells was observed. This increase was statistically significant ($P < 0.001$) and provides evidence that T cells participate in the induction of monocyte PCA in the rat.

DISCUSSION

The capacity of LPS to stimulate cells of the lymphoid system in an antigen-independent manner via its lipid A moiety so as to elicit various cellular responses is well known and includes the capacity to initiate and propagate extrinsic effector protease pathways such as the coagulation and fibrinolytic cascades. This has been demonstrated in a few species, including rabbits, mice, and humans (16, 17, 23, 26). The induction of PCA by endotoxin through cellular collaborative pathways has been demonstrated in mice and humans (16, 23), and in this study this cellular mechanism appears to be valid for rats as well. These studies provide a conceptually cohesive theme, in which T cells can serve as the recognitive unit in cellular pathways by which a wide variety of biologically important molecules (e.g., antigen, allogenic cells, viruses, or tumors) are able to elicit inflammatory responses from cells of the monocyte-macrophage lineage. Evidence exists also for direct stimulation by LPS of some cells of the monocyte-macrophage lineage (7, 17, 26). Whether this is an artifact of the isolation methods is not clear. Indeed, it now appears that multiple pathways exist, by which T cells may control the effector functions of monocytes and macrophages (T. S. Edgington, H. Helin, S. A. Gregory, G. Levy, D. S. Fair, and B. S. Schwartz, Proceedings of the Fourth Leiden Conference on Mononuclear Phagocytes, in press).

The response of the rat to LPS has been controversial. It has been claimed that rats do not respond with leukocyte PCA when stimulated with LPS, and this unresponsiveness might be the basis of the relative refractoriness of the rat to endotoxin-induced DIC in vivo (24, 25). An inherent absence of this response in the rat would represent a fundamental exception to mechanisms by which responses of cells of monocyte lineage are controlled. In this study we demonstrated that several rat strains do indeed respond in vitro to generate monocyte procoagulant activity, although the response is modest. The response was dose dependent, and the kinetics were similar to those described for the response of human cells to LPS and immune complexes (16, 22). PCA was expressed by monocytes, but T lymphocytes were required in a collaborative role for the full development of the PCA response, as has been described previously for humans and mice (15, 21). These studies involved the isolation of monocytes not directly responsive to LPS. Thus the rat does not appear to be an exception to the rule. The reason for the relative unresponsiveness of the rat when examined for in vivo DIC may reflect (i) a more effective clearing and catabolism mechanism of endotoxin as compared with that in other species, (ii) a more efficient fibrinolysis system, (iii) a relatively diminished initiation of the extrinsic coagulation pathway, and (iv) a requirement for

TABLE 6. T-cell and monocyte collaboration in the LPS-induced PCA

Stimulus[a]	PCA (mU/10^6 cells) in[b]:			
	PBM[c]	T lymphocytes[d]	Monocytes[e]	T lymphocytes[d] + monocytes[e] (ratio 10:1)
Medium	50 ± 4	33 ± 2	20 ± 2	36 ± 5
50 μg of LPS per ml	95 ± 5	29 ± 2	25 ± 4	54 ± 4

[a] 10^6 cells per ml were incubated for 5 h with or without stimulus.
[b] Mean ± SD.
[c] 8.7% esterase-positive cells.
[d] >99% esterase-negative cells; the cells were obtained from Fischer 344 rat PBMs by depletion of adherent cells on fibronectin-coated surfaces followed by nylon wool purification (see the text).
[e] 91% esterase-positive cells; the cells were obtained by adhesion to fibronectin-coated surfaces (see the text).

somewhat higher concentrations of endotoxin than those in mice or humans to elicit the PCA response.

We characterized the procoagulant effector molecule of the rat monocyte PCA. It was not dependent on vitamin K and it was not inhibited by serine protease inhibitors. The monocyte PCA did not accelerate the coagulation of factor X- and VII-deficient plasmas, indicating a requirement for these proteases in mediation of the activity, which is consistent with the presence of tissue factor. The LPS-induced rat PCA was not affected by iodoacetamide, indicating that the activity was not a cysteine protease as described by Gordon and Cross (11). We tentatively suggest that the cells express tissue factor as the predominant initiator of the coagulation protease cascade, thus being analogous to the human system.

Further analysis of the cellular responses of the rat to endotoxin may be informative as to the basis for the limited pathology of the response in vivo, which is information that may have a positive impact on the beneficial manipulation of the pathogenesis of disseminated intravascular coagulation in those species such as humans that are particularly susceptible.

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